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약학석사학위논문

Anoctamin 8 의 활성화와 생물 물리학적 특성 연구

The Activation and Biophysical Properties of Anoctamin 8

2018 년 8 월

서울대학교 대학원

분자의학 및 바이오제약학과

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박 세 라

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지도교수 신 영 기

이 논문을 약학석사학위논문으로 제출함

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ABSTRACT

ANO8 is a member of the Anoctamin/TMEM16 family which was previously defined as a superfamily of calcium-activated chloride channels after the identification of ANO1. However, it was found that the Anoctamin family consist of both cation and anion channels due to the identification of ANO9 as a non-selective cation channel. Among them, ANO8 shows low structural homology from the other Anoctamin members and it has not been well studied. Therefore, the aim of the research was to investigate not only the activation of ANO8 but also its biophysical properties.

The plasma membrane localization of ANO8 in HEK293T cells was visualised by immunofluorescent images. This led to examining the electrophysiological characteristics of ANO8 with whole-cell patch clamp recordings. It was unveiled that ANO8 was activated by intracellular cyclic adenosine monophosphate as well as high concentrations of calcium. Moreover, the cAMP-induced currents of ANO8

was enhanced by the addition of intracellular calcium.

The results from RT-PCR suggest that TMEM16H had an unusually high distribution in brain, spinal cord and dorsal root ganglion neurons. Immunostaining of dorsal root ganglion reveals ANO8 is highly expressed in nociceptive neurons.

Next, the function of ANO8 was examined by calcium imaging and it potentiated the responses of TRPV1 and TRPA1 channels that are involved in the transduction of noxious stimuli.

Taken together, this study reveals how ANO8 is activated and its other biophysical characteristics.

Keywords : TMEM16H/ANOCTAMIN8, cAMP, Calcium, Channel, Membrane protein

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INTRODUCTION

Ion channels are crucial to all living organisms as they are essential in the ability of excitable cells to convert chemical or mechanical stimuli into electrical signals. These excitable cells include neurons, muscle cell, touch receptor cells and the signals are sent to higher nervous system. Until now, enormous discoveries about different classes of ion channels have take place.

The Anoctamin/TMEM16 family is a group of ten mammalian transmembrane proteins of which most of their functions are yet to be defined. The family attracted great attention due to the identification of ANO1 and ANO2 as calcium-activated chloride channels (1-2). The Anoctamins gained more interest because of its implications in many physiological functions (3-4) as well as diseases including tumorigenesis (5), musculoskeletal disorder, prostate cancer, ataxia and so on (6-7). Especially, ANO1/TMEM16A, which is the most extensively studied member, was cloned in our laboratory and found to play an

important role in regulating secretion from epithelial cells(8). ANO1 was also reported to conduct heat sensation in nociceptive neurons (9).

ANO2 is also classified as a calcium-activated chloride channel that is associated with presynaptic photoreceptor terminal activities, olfaction (10) and synaptic response in hippocampal neurons (11).

ANO3 is known to interact with the sodium-activated potassium channels in dorsal root ganglion neuron, regulating sensory transduction (12). Moreover, scramblase activities have been detected from ANO6 (13) and it acts as a calcium-activated non-selective cation channel (14). The transmembrane segments of the Anoctamin family has also been studied as switching the TM5 compartments between ANO1 and ANO6 reduced the selectivity of ions that each of them are known to permeate (15).

Recently, from our laboratory, ANO9 was identified as a non-selective cation channel activated by cAMP and mainly found in colonic areas (16).

The expression of the Anoctamins across various tissues as

well as its varying physiological functions demonstrate the importance of the research on them. Unlike what was known previously, the members of the ANO family are not all CaCCs (Calcium-activated Chloride Channels), but consist of both anionic and cationic channels, proving its diverse nature.

ANO8 is most distant from the phylogenic tree of the Anoctamin family and it shows least structural homology to ANO1 among the other members. ANO8 has not been extensively studied before and its activation mechanism remained in question. Along with this, the high expression pattern of ANO8 in the central nervous system makes it interesting to study.

In this context, we strived to investigate if ANO8 is a channel, how it is activated and moreover, its other biophysical properties and potential physiological functions.

METHODS

1. Cell culture and transfection

HEK293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 10units/mL penicillin and streptomycin. The cells were maintained at 37℃, 5% CO_2.

The cells were transfected using mANO8 cDNA with FuGene HD (Roche Diagnostics) reagent. The experiments were performed 24-48 hours after transfection.

2. Immunofluorescence

ANO8-eGFP overexpressing HEK283T cells were grown and fixed on coverslips using 4% paraformaldehyde for 15mins in 4℃. The coverslips were blocked with 1% bovine serum albumin overnight in 4℃, then stained with Cell Mask dye (Thermofisher Scientific, 1:200) for 10mins

and counterstained with Hoechst (H3570, Thermofisher Scientific, 1:10000) for 5min, then visualized.

For tissue immunocytochemistry, the dorsal root ganglion tissue was extracted from 7-week old mice. The extracted tissue was embedded in OCT (Tissue-Tek) and 20㎛ cryostat sections were obtained. The glass slides were fixed with methanol for 15 mins at root temperature, followed by blocking with 1% bovine fetal serum, 5% goat serum and 0.2% triton X. The sections were incubated with the rabbit TMEM16H polyclonal antibody (1:200). For double staining, the slides were incubated with NFM (1:200, SantaCruz), TRPV1 (1:200, SantaCruz) or CGRP (1:200, SantaCruz) overnight at 4℃. The slides were washed using 1xPBS and stained with secondary antibodies conjugated with Alexa Fluor 594 or 488 (1:5000, ThermoFisher Scientific) for 1 hour at room temperature. After washing with PBS, it was counterstained with Hoechst (H3570, Thermofisher Scientific, 1:10000) and images were taken under confocal microscopy (Zeiss).

3. Whole-cell patch clamp recordings

For voltage-clamp experiments, whole-cell currents were recorded after formation of a gigaseal(>1GΩ) and ruptured the cell membrane with the patch electrode, making whole-cell configuration. The recordings were amplified with Axopatch 200B (Molecular Devices) and the obtained data were analysed with Clampfit 10.6.

The external solution contained (in mM) 140 NaCl, 2 $CaCl_2$, $MgCl_2$ and 10 HEPES. The pipette solution contained 140 $CsCl_2$, 2 $MgCl_2$, 10 EGTA and 10 HEPES, all adjusted to pH 7.2 and 300mOsm/L.

4. Calcium Imaging

ANO8-pEGFPN1 overexpressing HEK293T cells were grown on 8-well culture dish (ThermoFisher Scientific) and loaded with Fluo3-AM (Invitrogen) using 0.1% Pluronic F-127 (Invitrogen). After 30-45min incubation,

the cells were washed with HBSS (ThermoFisher Scientific) three times and used for experiment. Various chemicals diluted in HBSS were applied to the cells and measured at 488 nm every 3 seconds with confocal microscopy (LSM700, Zeiss).

5. Total RNA purification and first cDNA synthesis

Mouse tissue organs were isolated with 7-week old C57/B6 male mice. Total tissue RNAs were extracted using the EasySpin Total RNA Extraction Kit (iNtROn Biotech) according to the manufacturer's protocol. The first strand cDNAs were reversely transcribed using GoScript (iNtROn Biotech) according to the manufacturer's protocol.

6. Reverse Transcriptase Polymer Chain Reaction

With cDNAs obtained from reverse transcription, RT-PCR

was performed using DreamTaq (ThermoFisher Scientific). The primers used were; GAPDH forward 5’-GTGAAGGTCGGTCTCAACGG-3’ and reverse 5’-CCCATCACAAACATGGGGGC-3’ and ANO8 forward 5’-CTTGGAGGACCAGCCAATC-3’ and reverse 5’-TGAACTGGAAACACCTGCTG-3’.

7. Statistical Analysis

The data obtained from all experiments were presented in mean ± standard error of more than five independent experiments. Statistical differences were analysed using unparied t-test between groups, where p value less than 0.05 is statistically significant.

RESULTS

1. Overexpression of ANO8 in HEK293T cells

In order to examine the cellular localization of ANO8, mANO8-pEGFP-N1 vector was overexpressed in HEK293T cells. ANO8 was expressed in the plasma membrane and it was also co-localized with CellMask dye which is the plasma membrane marker (Figure 1).

2. Activation and other electrophysiological properties of ANO8

As to investigate what activates ANO8, various chemicals were applied to ANO8-overexpressing HEK293T cells using whole-cell patch clamp technique. Most of them failed to activate ANO8, however, 100μM of cAMP in intracelluar pipette solution induced inward currents (87.4

± 6.03pA/pF, n=10) at E_{hold} = -60mV. These currents were not observed in HEK293T cells expressing empty vector pcDNA3.1, which was used as a control (figure 2). Next, ANO8-overexpressing HEK cells were treated with 2mM H-89 (known as a PKA blocker) for 10minutes before the experiment, cAMP currents were completely abolished (figure 3).

3. Intracellular Calcium augments cAMP-induced currents of ANO8

In order to investigate if intracellular calcium activates ANO8 as well, various concentrations of calcium was added to the pipette solutions. 20μM of intracellular calcium failed to evoke any currents from ANO8-HEK cells, however, when 40μM of calcium was applied to the pipette solution, inward currents (38.9 ± 5.51 pA/pF, n=10) were observed from ANO8-HEK cells during whole-cell recordings. The control HEK cells showed no appreciable currents induced by intracellular calcium

(figure 4).

10μM Calcium failed to evoke whole-cell currents from ANO8-transfected HEK cells and 50μM cAMP also only induced small currents. Therefore, to investigate if intracellular calcium has an effect on cAMP-evoked currents of ANO8, we applied 10μM Calcium and 50μM cAMP together in the pipette solution. From whole-cell recordings, it was revealed that with intracellular calcium, cAMP-induced currents were largely augmented in ANO8-HEK cells (figure 5). In addition, ANO8 was activated for a longer period compared to it being activated by cAMP alone.

4. ANO8 is not activated by voltage alone

To see if voltage also activates ANO8, the voltage steps from -100mV to +100mV in 10mV increment were applied to control and ANO8-transfected HEK cells. The cells were treated with voltage-gated potassium channel

blocker, 4-aminopyridine to block the effect of these channels. No difference in I-V curves between the control and ANO8 cells highlights that voltage alone does not activate ANO8 (figure 6).

5. ANO8 is permeated by cations

To examine which ions are responsible for the ANO8 currents, the sodium HEPES bath solution was replaced to NMDG (N-methyl D-glutamine)-Cl bath solution. With NMDG-Cl bath, cAMP failed to evoke currents in ANO8-overexpressing HEK cells (figure 7).

Furthermore, using 140mM KCl pipette solution, the bath solutions were changed from 70mM to 210mM KCl with 100μM cAMP. During the activation of ANO8, voltage ramps from -100mV to +100mV were applied for 100ms. The reversial potential(E_{rev}) which was initially 0mV at 140mM KCl bath solution, changed to +11.9 $\pm$ 0.91mV at 210mM and -8.33 $\pm$ 1.39mV (n = 4) at 70mM (figure 8).

6. Tissue distribution of TMEM16H and its expression pattern in dorsal root ganglion neurons

Expression profiles of ANO8 were determined by RT-PCR with various mouse tissue samples including (certain brain regions, spinal cord, dorsal root ganglia, skin, skeletal muscle, liver, stomach, heart, pancreas, spleen, retina and intestine). ANO8 showed an unusual high expression in central nervous system as well as dorsal root ganglia (figure 9). This gives implications in the possible role of ANO8 in the central nervous system such as sensory transduction.

After examining the expression profile of ANO8, we performed an immunocytochemical analysis in dorsal root ganglia. A variety of antibodies which are a marker for diverse subsets of neurons, was used. ANO8 immunoreactivity was detected in almost all dorsal root ganglion neurons (figure 10). ANO8 was also co-localized with NFM (a marker for mylinated DRG neurons), CGRP (a marker for peptidergic small sensory neurons), IB4 (a marker of non-peptidergic small sensory neurons) and

TRPV1 (norciceptor marker).

7. Effects of ANO8 on TRP channels involved in sensory transduction in HEK293T cells

As to examine whether ANO8 has any effect on activity of sensory channels, we co-transfected ANO8 with TRP channels known to be involved in sensory transduction and performed calcium imaging. For the control group, HEK293T cells were transfected with pcDNA3.1.

TRPV1 channel was co-transfected with ANO8 in HEK293T cells and tested for difference in levels of calcium influx. Compared to TRPV1 being transfected without ANO8, the capsaicin-induced calcium influx was elevated in co-transfected HEK cells by 30.5% on average (figure 11).

Another channel involved in sensory pathway expressed in dorsal root ganglia, TRPA1, was also co-transfected and tested. An elevation of calcium influx was again observed after application of allyl isthiocyanate 100μM in the

co-transfected HEK cells, in comparison to HEK cell expressing both TRPA1 and pcDNA (figure 12).

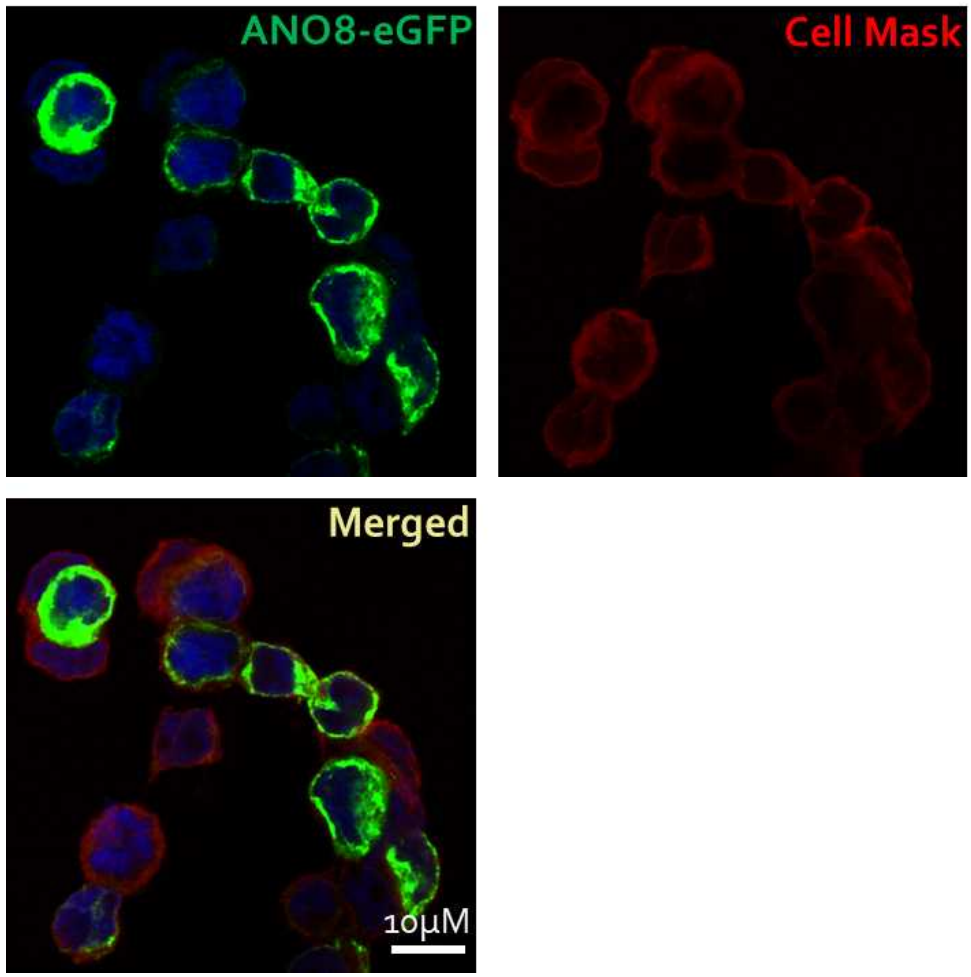

Figure 1. The cellular localization of ANO8

HEK293T cells were overexpressed with mANO8-pEGFPN1 and merged with CellMask, the plasma membrane marker.

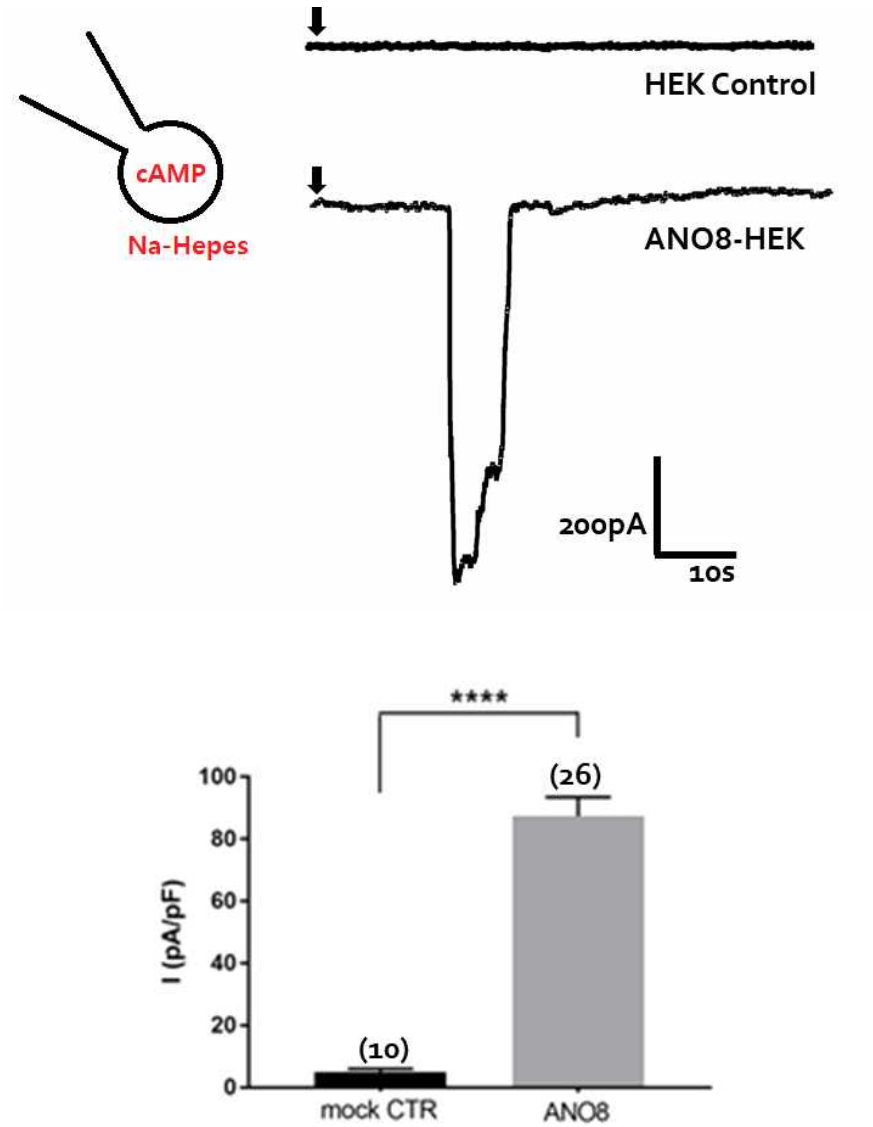

Figure 2. Intracellular cAMP activates ANO8

Representative whole-cell currents of ANO8-transfected HEK293T cells activated with 100 μM cAMP and summary of cAMP-induced currents compared with the control.

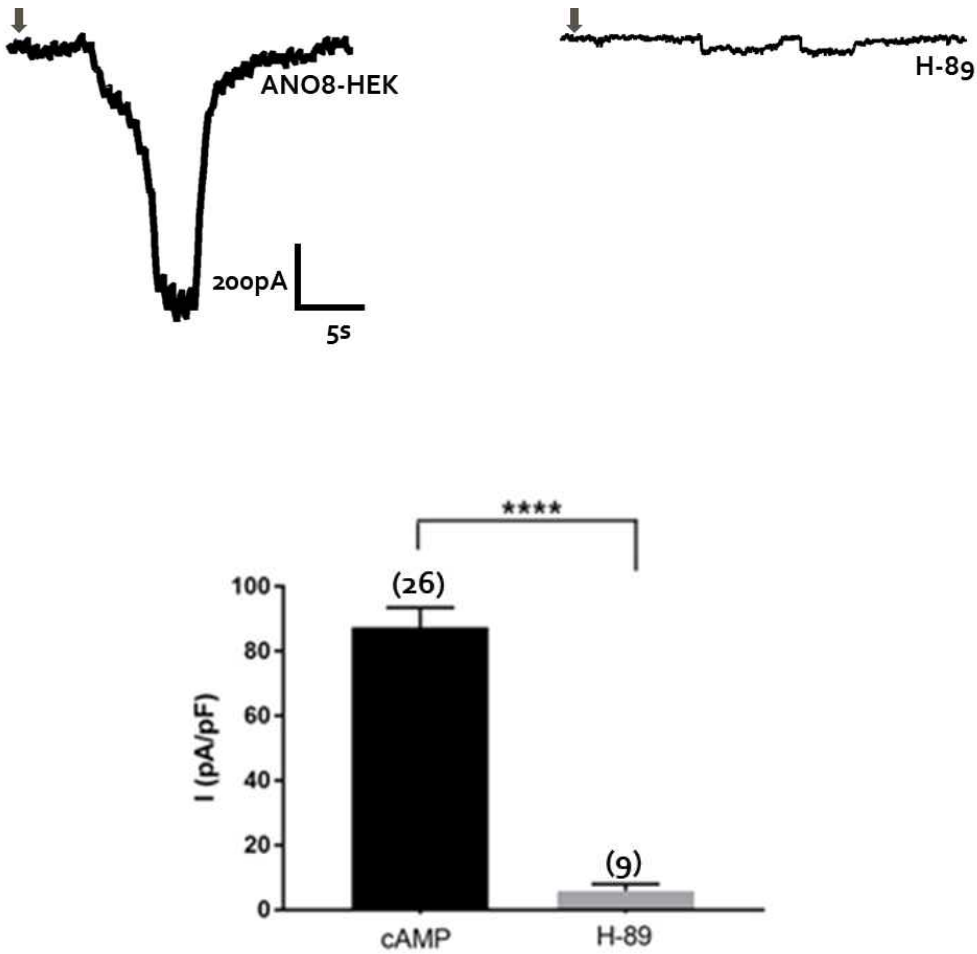

Figure 3. H-89 blocks cAMP-activated currents of ANO8

Pre-treatment of H-89 completely abolishes the inward currents of ANO8, activated by intracellular cAMP.

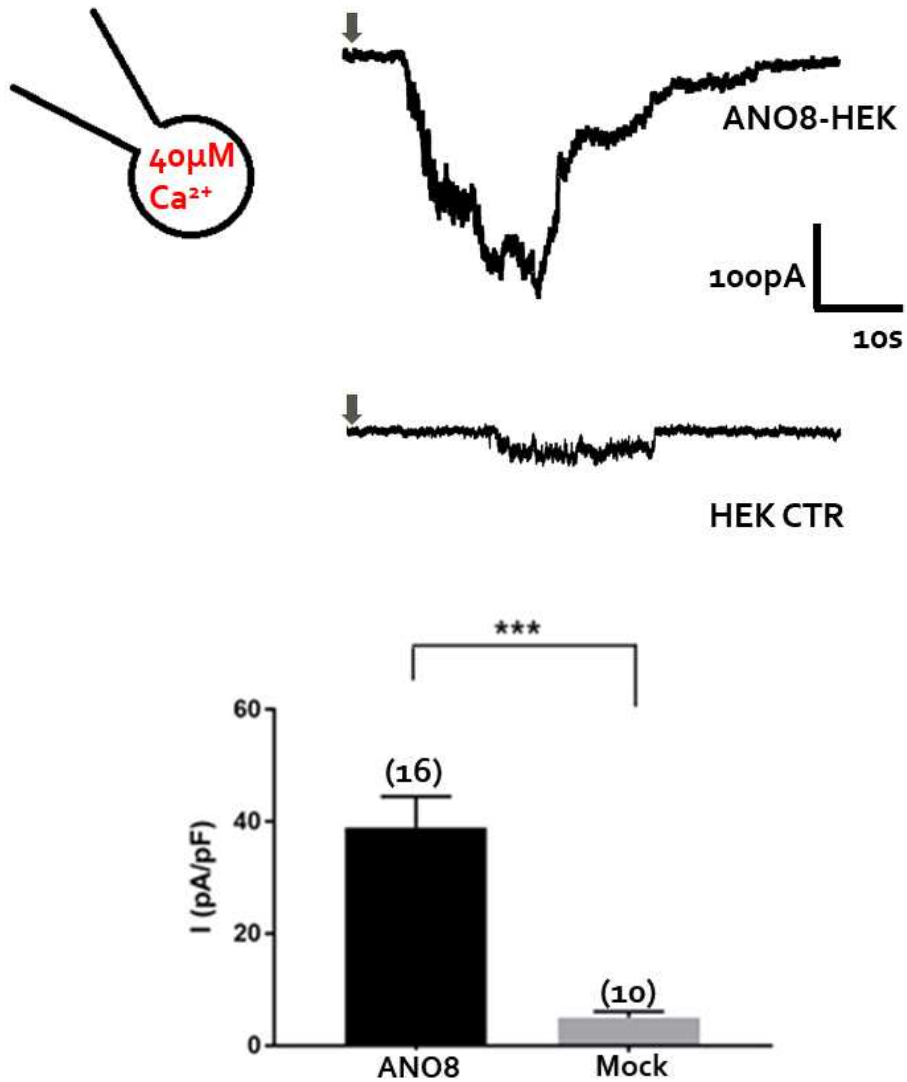

Figure 4. Intracellular calcium activates ANO8

Whole-cell current traces of ANO8-HEK293T cells activated with 40 μM intracellular calcium.

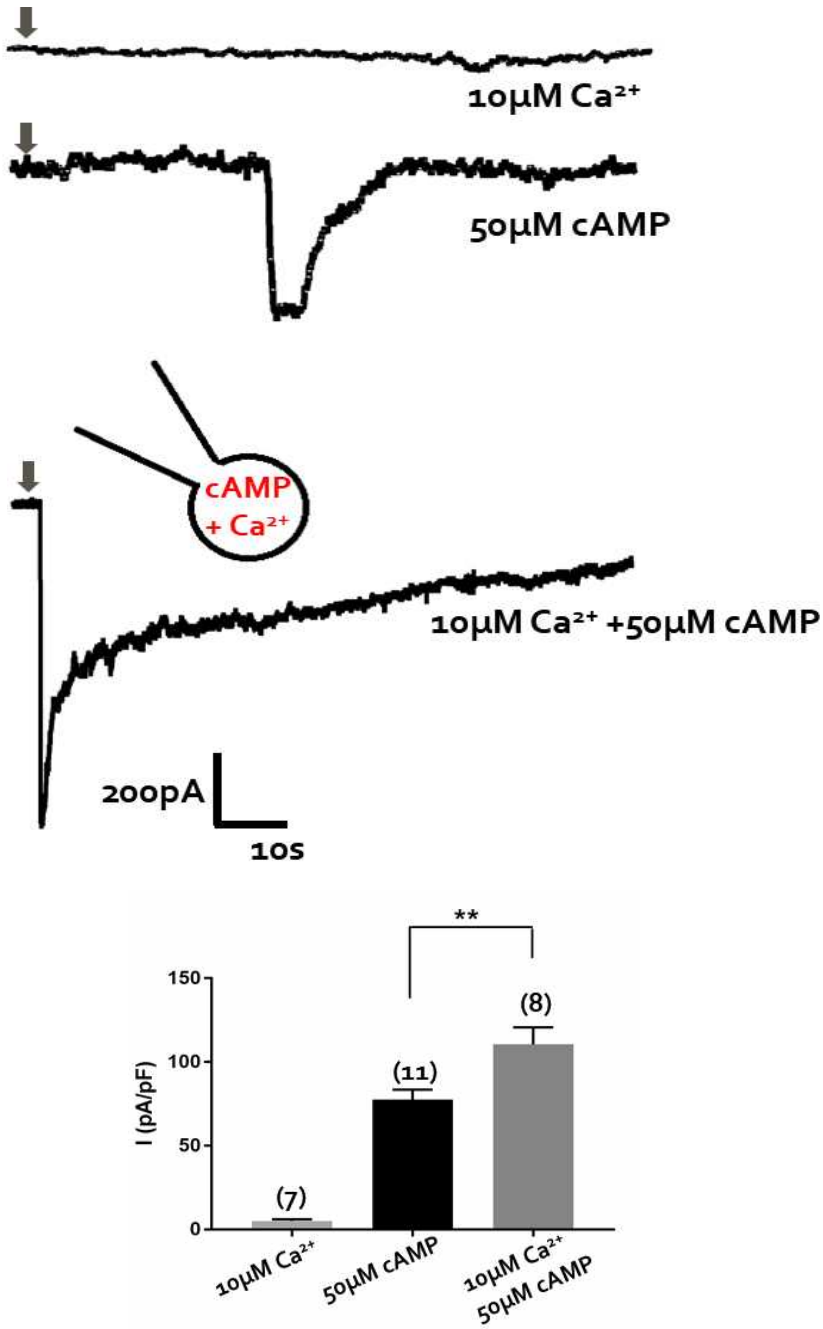

Figure 5. Intracellular calcium amplifies cAMP-evoked currents of ANO8

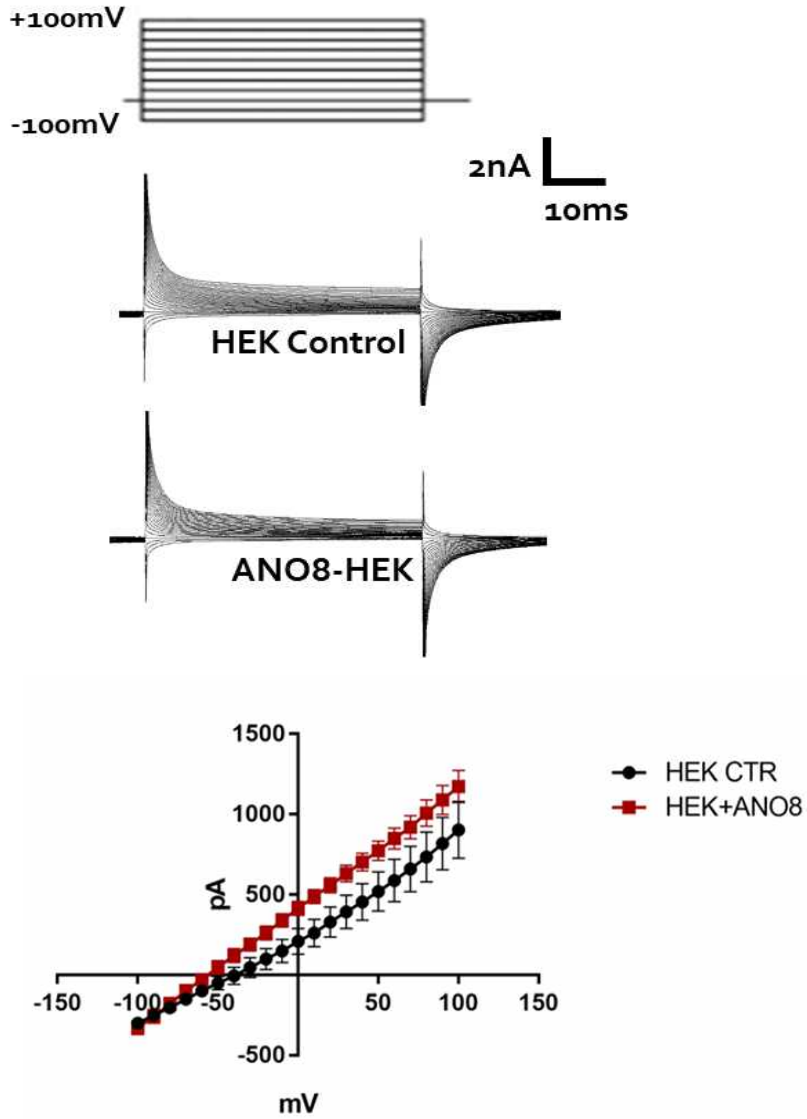

Figure 6. Voltage independence of ANO8

Whole-cell currents of ANO8 transfected cells and the control, voltage steps from -100mV to +100mV were applied.

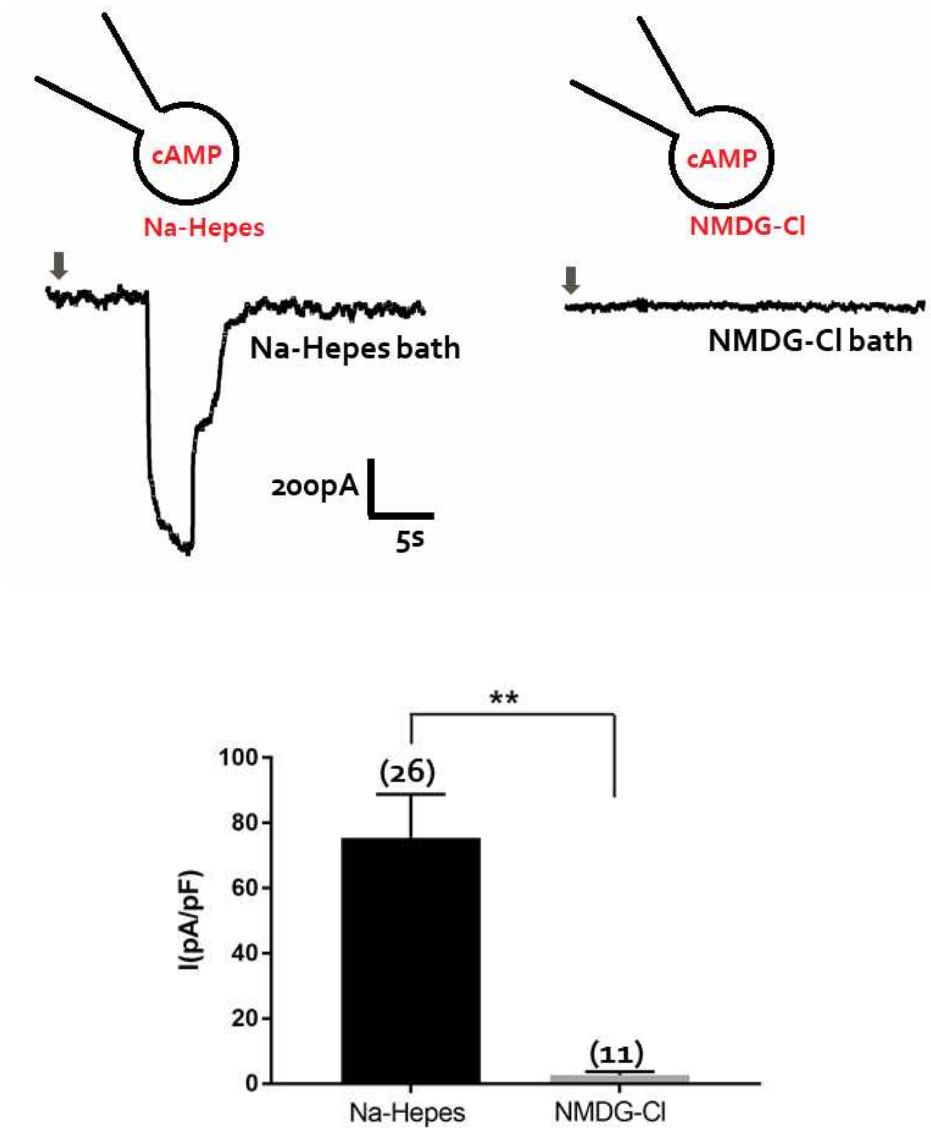

Figure 7. NMDG-Cl bath blocks cAMP currents of ANO8

The replacement of Na-Hepes to NMDG-Cl bath solution blocks ANO8 currents activated by intracellular cAMP.

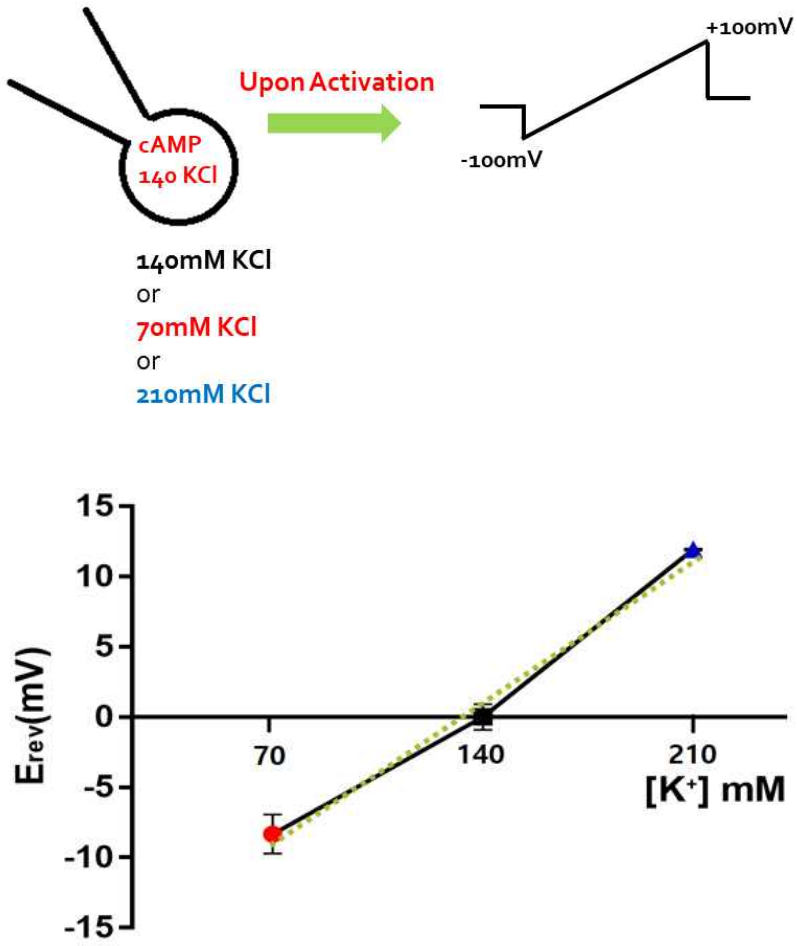

Figure 8. ANO8 is permeable to cations

The reversial potentials were measured when the extracellular solutions were changed from 70mM to 210mM KCl solutions during the activation of ANO8.

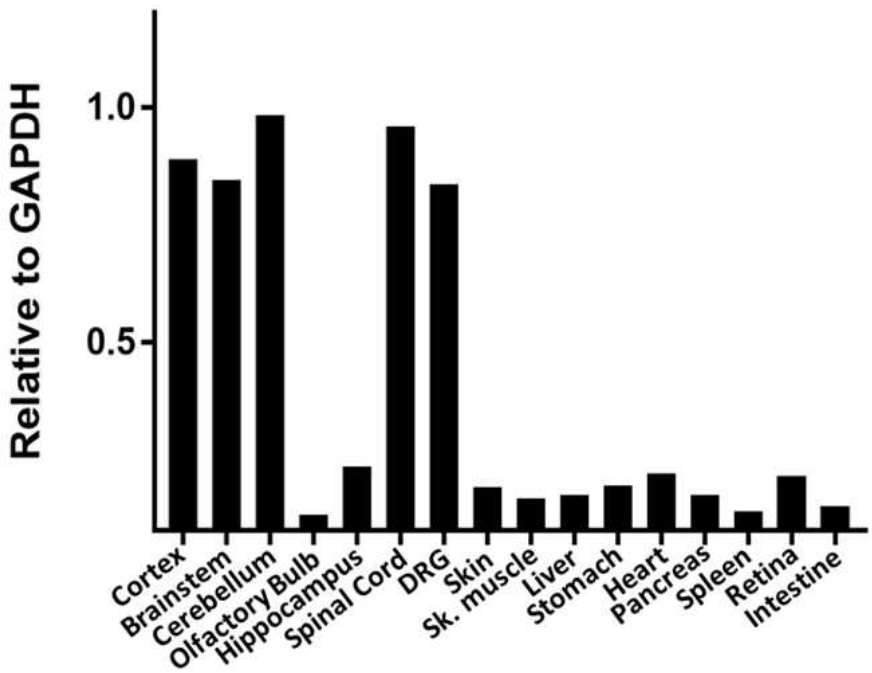

Figure 9. Tissue distribution of ANO8

mRNA transcript levels of ANO8 in various mouse tissue organs.

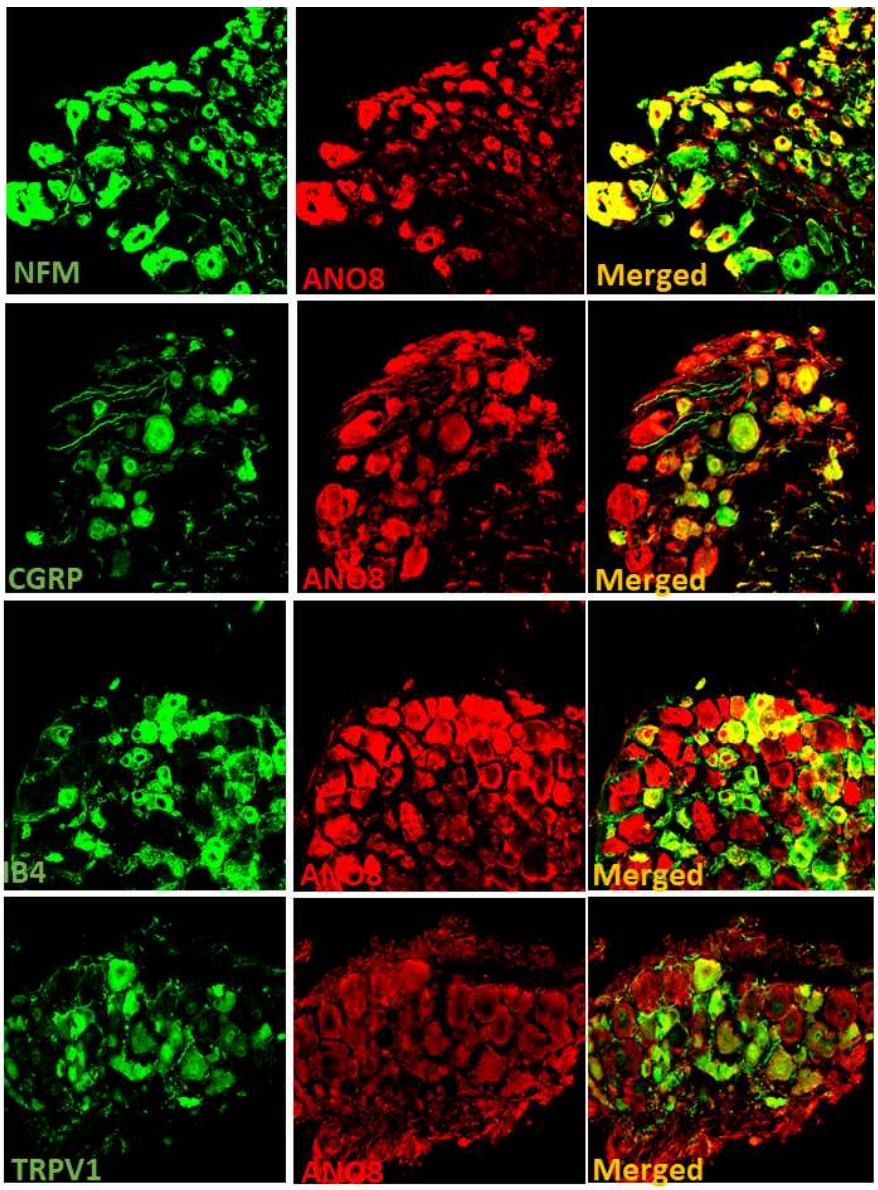

Figure 10. Expression of ANO8 in dorsal root ganglia

Immunoreactivity was detected for ANO8 and colocalised with NFM, CGRP, IB4 and TRPV1 (all diluted in 1:200).

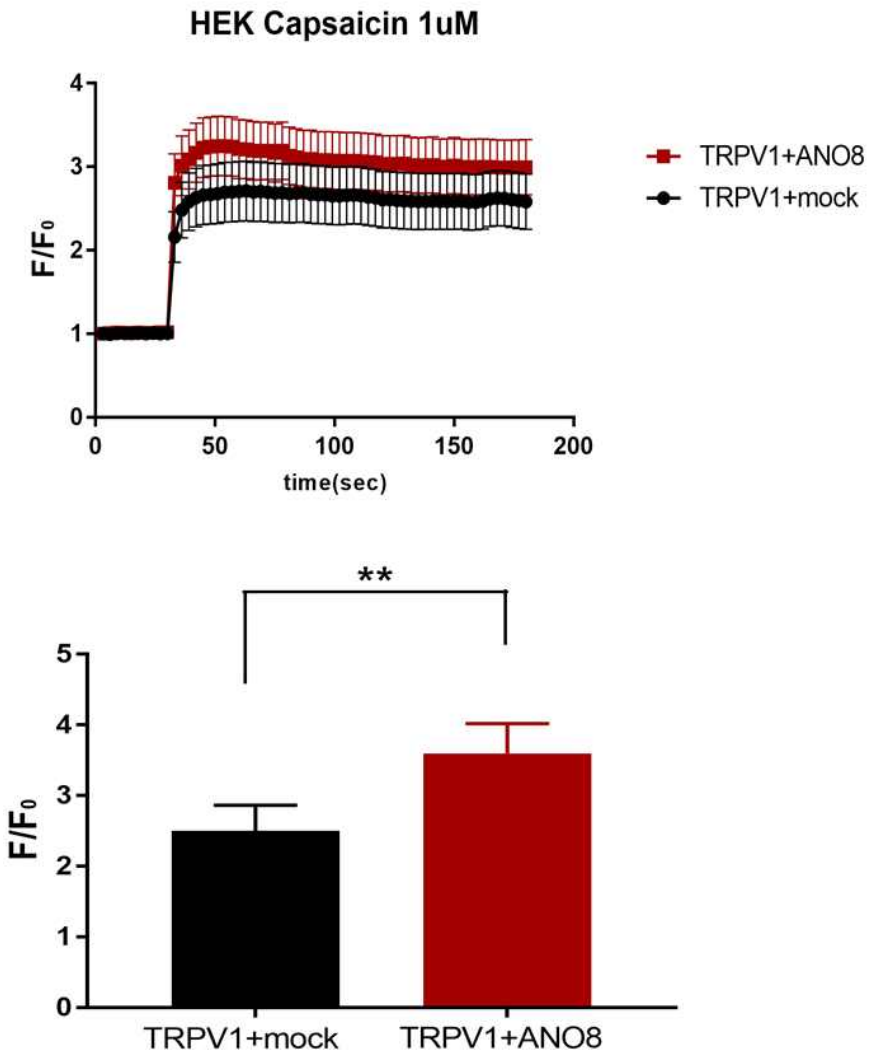

Figure 11. ANO8 potentiates TRPV1 response

TRPV1-mediated calcium influx is elevated in ANO8 and TRPV1 co-transfected HEK293T cells.

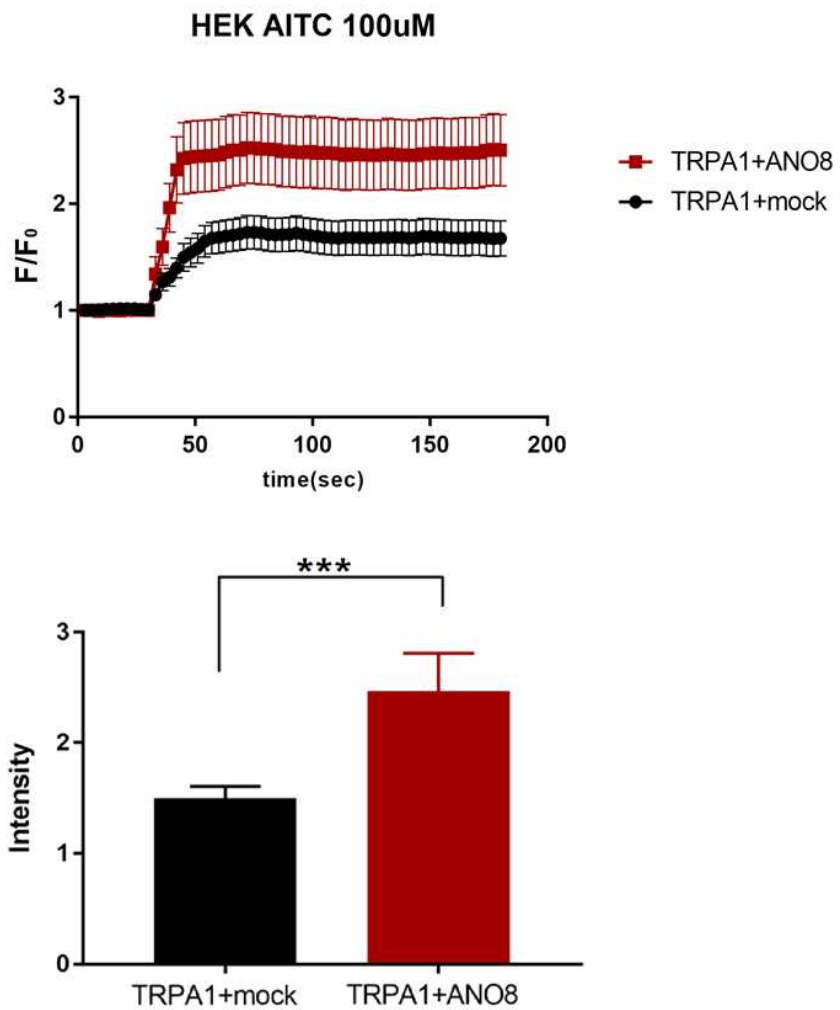

Figure 12. ANO8 potentiates TRPA1 response

TRPA1-mediated calcium influx is increased in ANO8 and TRPA1 co-expressing HEK293T cells.

DISCUSSION

The Anoctamin family is a group of 10 membrane proteins in mammals with diverse tissue distribution profiles and various physiological roles as some members are implicated in cancers and other disorders (3,7). The Anoctamins have attracted great attention after the identification of ANO1 and ANO2 as calcium-activated chloride channels (1,2). The excitement about the Anoctamin family continued as ANO1 was found to be involved in nociception (9) and tumorigenesis, and also, ANO2 had a role in olfactory sensation (4) as well as synaptic functions in hippocampus (10). Other members such as ANO3 is known to modulate nociception in dorsal root ganglia (12) and the mutation of ANO5 is associated with muscular dystrophy (17). ANO6 is known to be involved in Scott syndrome (18) and ANO10 is linked with autosomal recessive spinocerebellar ataxia (19). All these findings stress the importance of the Anoctamin family in

crucial physiological processes, so it is worthwhile to study.

Although the Anoctamins were previously known as CaCCs, upon the identification of ANO6 and ANO9 as non-selective cation channels (16), it is clear that the family consists of both anion and cation channels. Despite the great interest towards the Anoctamins, the activation mechanisms of many members are mostly unveiled.

ANO8 is a member showing low degree of homology from the other Anoctamin members and its activation has remained in question. The aim of the present study was to investigate what activates ANO8 and what other biophysical properties ANO8 would possess.

The results demonstrate intracellular cAMP indeed activates ANO8. The pre-treatment of H-89 (PKA blocker) abolished ANO8 currents induced by cAMP, confirming the activation of ANO8 is cAMP/PKA pathway dependent.

Intracellular calcium, not as robust as cAMP, also elicited inward currents of ANO8. However, the amount of calcium

used was extremely high and usual physiological concentrations of calcium failed to activate ANO8. Surprisingly, when calcium was added together with cAMP intracellularly, this enhanced the activation of ANO8. Compared to cAMP-evoked currents of ANO8, the additional application of calcium prolonged the activation time of ANO8. Although calcium alone is not critical, intracellular calcium with cAMP seem to act synergistically in the activation of ANO8.

Replacing the NaCl base bath solution to NMDG-Cl bath blocked the cAMP-evoked currents of ANO8. As NMDG substitutes for sodium ions and disturb the influx of cations (mostly sodium ions), the main ions permeating ANO8 are cations. This results were further confirmed by the shift in reversial potentials when the extracellular solutions were changed from 70mM to 210mM and 100ms voltage ramps were applied. The reversial potentials were plotted and the best line of fit resembles the Nernst equation when the major permeating ions are cations. Therefore, the main ions gated by ANO8 are cations rather

than anions.

The expression profile of ANO8 demonstrates its high distribution in specific brain regions (cortex, brainstem, cerebellum), spinal cord and dorsal root ganglia. The peculiar expression pattern of ANO8 indicates its importance in the central nervous system as well as dorsal root ganglia. Especially, ANO8 was found ubiquitously and robustly in the neuronal cells of dorsal root ganglia. ANO8 was expressed in small- to large-diameter neurons as well as peptidergic to non-peptidergic neurons. ANO8 also co-localized with nociceptive marker. As the expression of ANO8 is omnipresent in all subsets of DRG neurons, this increases the importance of ANO8 in DRG functions.

Moreover, TRP channels are a group of non-selective cation channels and are highly expressed in dorsal root ganglion neurons (20). They play critical roles in temperature and sensory transduction. The chemical or mechanical stimuli are received through these channels are transformed to action potentials in dorsal root ganglia, consequently sent to higher nervous system (21). As ANO8

enhanced TRPV1- and TRPA1-mediated calcium influx, this may imply the possible role of ANO8 in sensory transduction such as pain and itch.

In conclusion, the activation and biophysical properties of ANO8 were examined in this study. ANO8 is discovered to be activated by intracellular cAMP and its activation is indeed cAMP/PKA dependent. The tremendous ANO8 expression in the central nervous system and its omnipresence in every dorsal root ganglion neurons provide implications in the potential physiological role of ANO8.

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국 문 초 록

ANO8은 세포 내 칼슘이온에 의해 활성화 되어 ANO1의 발견 이후 염소이온을 통과시키는 이온채널 가족으로 분류되었던 Anoctamin/TMEM16 과 구성원 중 하나이다. 비선택적 양이온 채널인 ANO9의 발견 이후에 아녹타민들은 양이온과 음이온 채널들이 함께 존재하는 이온채널들임이 밝혀졌다. 그 중, ANO8은 다른 아녹타민들과 비교하여 가장 낮은 구조적 유사성을 보였고, 현재까지 많은 연구가 알려지지 않았다. 본 연구의 목적은 ANO8의 활성화와 생물 물리학적 특성에 대해 밝히는 것이다.

ANO8의 HEK293T 세포 막 발현을 형광항체 기법으로 (immunofluorescent microscopy) 확인했고, 이 결과를 바탕으로 ANO8을 패치 클램프 기법(whole-cell patch clamp)을 통해 전기생리학적 특성을 연구했다. ANO8은 cAMP에 의해 활성화가 되는 것으로 밝혀졌고, 높은 칼슘 또한 ANO8을 활성 시키는 것으로 밝혀졌다. 또한, cAMP에 의해 생긴 ANO8의 전류는 세포 내 칼슘에 의해 증폭되었다.

쥐 세포 조직들의 중합 효소 연쇄 반응을 통해 ANO8이 뇌, 척수, 후근신경절 뉴런 (Dorsal root ganglion)에 대단히 높은 분포를 보였다.

후근 신경절 조직의 항체형광염색에서 ANO8이 모든 후근 신경절 뉴런들에 구분 없이 존재한다는 것을 밝혔다.

칼슘 이미징 기법(calcium imaging)을 이용해 ANO8이 유해한 감각 전달에 관여하는 TRPV1과 TRPA1 이온 채널들의 역할을 증대시키는 역할을 확인했다.

결론적으로, 본 연구는 ANO8이 세포 내 고리형 아데노신 일인산(cAMP)에 의해 활성화 되고 ANO8의 활성화 기작은 인산염 키네이스 A (PKA)에 의존적이라는 것을 밝혔다. 또한, ANO8에 의해 통과되는 이온들은 주로 양이온들임 또한 알아내었다. 위 결과들과 더불어 ANO8의 높은 중추신경계와 척수 후근 신경절 뉴런들의 분포도는 중요한 생리학적 역할을 할 것임을 시사한다.

주 요 어 : TMEM16H/ANOCTAMIN8, cAMP, Calcium, Channel, Membrane protein

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